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Kickstarting Foxp3 with c-Rel

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In this issue of *Immunity*, reports by Long et al. (2009) and Ruan et al. (2009) suggest that the transcription factor NF-κB-c-Rel is an important molecular mechanism by which T cell receptor-specificity for self-antigens instructs the selection of Foxp3$^+$ regulatory T cells.

The selection of a small portion of developing T cells into the regulatory T (Treg) cell subset is a crucial step in ensuring that the immune system remains tolerant to self. Although T cell receptor (TCR) recognition of self-antigens has long been suggested to be important for this process, the molecular mechanism by which TCR activation leads to the expression of Foxp3, a transcription factor required for Treg cell stability and function, was unclear. In this issue of *Immunity*, reports by Long et al. (2009) and Ruan et al. (2009) demonstrate that this missing link is likely provided by NF-κB signaling via the c-Rel transcription factor.

Initial studies of TCR transgenic models demonstrated that thymic Treg cell development is dependent on interactions with cognate antigen, implying that self-antigen recognition is crucial for Treg cell selection. These data were further supported by the observation that Treg cells typically used different TCRs than conventional Foxp3$^-$ T cells in studies of mice with limited TCR diversity. However, the role of TCR specificity in Treg cell development has been controversial in recent years, based on data suggesting that events at the thymic double-negative (CD4$^-$CD8$^-$) stage before TCR rearrangement affected Treg cell development (Pennington et al., 2006), as well as the provision of an alternative interpretation of TCR repertoire studies (Pacholczyk et al., 2007). In part, this controversy persisted because of the lack of transgenic models of Treg cell development using naturally arising Treg cell TCRs, which was recently addressed by two independent groups (Bautista et al., 2009; Leung et al., 2009). The long delay in generation of these Treg cell TCR transgenic models was due to the fact that the selection niche for these Treg cell TCRs were remarkably small, such that Treg cell TCR transgenic mice harbored an extremely low frequency (<0.1%) of Foxp3$^+$ thymocytes, making it unclear whether this represented TCR-driven Treg cell development. As the clonal frequency of the TCR transgenic cells was decreased, the frequency of Foxp3$^+$ cells increased substantially. Importantly, TCRs from non-Treg cells were unable to facilitate Treg cell development under the same conditions, strongly supporting the original notion that Treg cell selection is dependent on TCR-derived signals.

Differences in TCR usage between Treg and conventional T cells could arise before or after induction of Foxp3. It has been argued that stochastic expression of Foxp3 allows those cells to survive negative selection, thus skewing self-reactive TCRs toward the Treg cell population (Van Santen et al., 2004). However, no Foxp3$^+$ cells were observed in several TCR transgenic lines (Bautista et al., 2009; Leung et al., 2009), even at low clonal frequency, suggesting that stochastic induction of Foxp3 does not occur, at least at readily detectable numbers. Thus, understanding the molecular mechanism by which certain TCR specificities were preferentially found in the Treg cell subset would be crucial for understanding thymic Treg cell development.

One potential pathway downstream of TCR has been suggested to be NF-κB, as mice deficient in upstream components such as PKC-θ, Bcl10, CARMA1, and MALT1 had few Treg cells (Figure 1). To carefully analyze the contribution of this pathway for Treg cell development, Long et al. utilized transgenic mice engineered to augment or inhibit NF-κB signaling (Long et al., 2009). NF-κB signaling involves receptor-mediated activation of the kinase IKK, which phosphorylates cytoplasmic IκB (inhibitor of κB), resulting in its degradation, thereby allowing the release of bound transcription factors to the nucleus. Therefore, NF-κB signaling can be enhanced by a constitutively active IKKβ kinase and inhibited by a nondegradable IκB ("+" and "−" in Figure 1, respectively). Here, they observed that enhancement of NF-κB signaling resulted in a 5-fold increase in the frequency of Foxp3$^+$ cells in the thymus, and for inhibition, a 50% decrease. They also observed that transgenic enhancement of NF-κB signaling can rescue the defect in CARMA1- and TAK1-deficient mice, demonstrating that NF-κB, and not other signaling proteins JNK or NFAT, is the relevant pathway downstream of the CARMA1-Bcl10-MALT1 complex. One curious observation was that Foxp3$^+$ cells elicited directly by NF-κB signals often did not express CD25 and were less suppressive compared with normal Treg cells. However, the Foxp3 locus in transgenic NF-κB-induced Treg cells was appropriately demethylated. Thus, although transgenic NF-κB-enhanced signals alone may not mimic all facets of Treg cell development; it is clear that NF-κB plays a crucial role.

To address whether NF-κB itself is sufficient for inducing Foxp3 without specific TCR signals, Long et al. studied OTII and P14 TCR transgenic mice in which those TCRs are unable to facilitate Treg cell development. Similar to the results with the upstream mediators CARMA1 and TAK1, transgenic enhancement of NF-κB signaling was also able to bypass the requirement for TCR self-recognition to induce Treg cell development. In fact, CD8$^+$ Treg cells were even observed at high frequency in the P14 TCR transgenic line. These data therefore suggest that NF-κB is a direct link between TCR

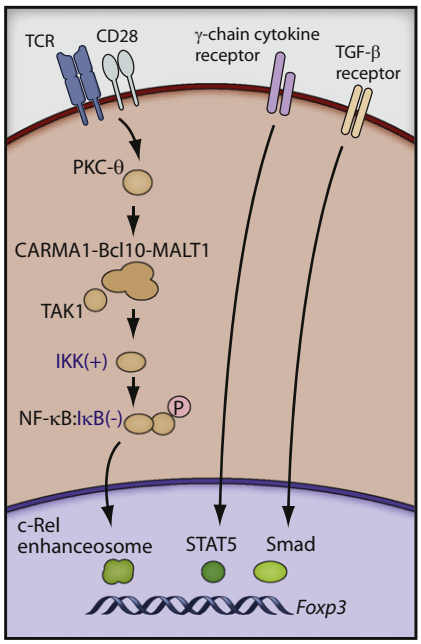

Figure 1. Simplified Representation of Foxp3 Induction

TCR and CD28 stimulation results in a signaling cascade that includes the CARMA1-Bcl10-MALT1 complex and TAK1 kinase, which leads to activation of IKK via phopshorylation. IKK then phosphorylates IκB, which is degraded and releases NF-κB transcription factor c-Rel to bind to the Foxp3 promoter. Long et al. (2009) utilize a constitutively active form of IKKβ to enhance NF-κB signaling, or a nondegradable form of IκB, which are indicated by a (+) or (–), respectively. Smad and Stat5 mediators of TGF-β and common γ-chain cytokines (IL-2, IL-15, and IL-7 in this case), respectively, are also shown.

specificity and selection into the Treg cell subset.

There are many potential mechanisms by which NF-κB could induce Foxp3. It could be direct by binding of NF-κB family transcription factors to the Foxp3 locus, or it could be indirect by affecting other genes important for Treg cell development such as IL-2. For example, it was recently reported that Stat5 played an important role in the induction of Foxp3, as transgenic expression of a hyperactive form of Stat5 markedly increased the frequency of Foxp3$^+$ cells (Burchill et al., 2008). However, neither the expression of common γ-chain receptors, nor sensitivity to the cytokines, was altered in with transgenic enhancement of NF-κB signaling. These data, therefore, argued against an indirect role of NF-κB in Treg cell development.

To determine if NF-κB directly played a role in the induction of Foxp3, Ruan et al. (2009) studied mice deficient in members of the NF-κB family, observing that c-Rel, and not p50 or RelB, were required for efficient development of thymic Treg cells. The presence of normal thymocytes was unable to reverse the inability of c-Rel- deficient cells to generate Treg cells in mixed bone marrow chimeras, further supporting a direct role of c-Rel in Foxp3 induction. Analysis of the Foxp3 promoter in vitro using luciferase assays revealed two NF-κB binding sites, which were confirmed using gel-shift assays. Chromatin immunopreciptation (ChIP) and sequential ChIP was then used to identify the transcription factors c-Rel, p65, NFATc2, and later, Smad and CREB at the promoter after TCR stimulation. Thus, these data demonstrate that TCR signaling results in the formation of a c-Rel enhanceosome in the Foxp3 promoter.

There are a couple of differences between these reports. Long et al. suggest that c-Rel binds as a dimer with p50 and can also bind to a region of Foxp3 distal to the promoter termed CNS3, whereas Ruan et al. suggest that c-Rel dimerizes with p65 and did not identify other binding sites for c-Rel. This could be related to differences in stimulation conditions or cell types used for their analyses. Nonetheless, both reports agree on a central role for c-Rel in Foxp3 transcription.

In the context of a recently proposed model for thymic Treg cell development (Burchill et al., 2008; Lio and Hsieh, 2008; Wirnsberger et al., 2009), these studies suggest that TCR signaling via NF-κB and c-Rel could provide an instructive signal to open the Foxp3 locus (Figure 1). Very strong TCR signals would still lead to negative selection by NF-κB and other pathways (Long et al., 2009). Common γ-chain cytokines, such as IL-2, would then induce high Foxp3 expression via Stat5, completing Treg cell development. TGF-β signals may also contribute, as deficiency in both TGF-β and IL-2 signals result in a dramatic decrease in thymic Treg cell frequency in adult mice compared with either one alone (Liu et al., 2008). Future studies will be required to address the requirement for a second cytokine-derived signal in the context of NF-κB-c-Rel for Foxp3 gene regulation in the thymus.

Regardless, this model is likely to be incomplete. Many pathways can induce NF-κB, and many cell types can receive Stat5 and Smad signals, yet high Foxp3 expression is primarily found on Treg cells. Additionally, it could be argued that induction of Foxp3 via NF-κB resulting from proinflammatory Toll-like receptor ligands or cytokines may be counterproductive to effective immunity against pathogens. The proposal by Long et al. that NF-κB is a thymic sensor of inflammation needs to be tested more rigorously. The observed relative preservation of Treg cell numbers after lipopolysaccharide challenge could have alternative explanations. For example, corticosteroid-mediated apoptosis could preferentially spare thymic Treg cells as they are more mature than most CD4$^+$ thymocytes. Taken together, it is likely that additional factors are involved in the regulation of Foxp3. Although future studies are required, the recognition that NF-κB-c-Rel links TCR specificity and Foxp3 expression is important for understanding the mechanism by which a few thymocytes are instructed, based on thymic antigen recognition, to become the regulators defending against home grown perpetrators of autoimmunity.

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